{Exhibit 41}

Rudkin and Stollar, "High Resolution Detection of DNA-RNA Hybrids in situ by Indirect Immunofluorescence," Nature, 265: 472-73 (1977)

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High resolution detection of DNA–RNA hybrids *in situ* by indirect immunofluorescence

We describe here a new method for the detection of RNA·DNA hybrids in cytological preparations with which we have revealed the locations of hybrid molecules on polytene chromosomes. The critical reagent is an antiserum raised in rabbits against poly(rA)·poly(dT) complexed with methylated bovine serum albumin, originally described by Stollar[1]. The specificity and resolving power of the indirect immunofluorescence procedure are demonstrated using *in situ* hybridisation of 5S rRNA (ribosomal RNA) to polytene chromosomes of *Drosophila melanogaster* as a model system. The method has significant advantages over the autoradiographic procedures[2–5] used so far.

The procedure for visualising the *in situ* hybrids follows Alfageme *et al.*[6] It consists of exposing the cytological preparation to the rabbit anti-hybrid antiserum, then to anti-rabbit IgG prepared in goat and tagged with rhodamine, followed by examination in a fluorescence microscope (see legend to Fig. 1). Our test objects were polytene chromosomes of *Drosophila melanogaster* (giant phenotype) to which 5S rRNA had been hybridised *in situ* (see legend to Fig. 1). The two glands from a single larva either were used as duplicate samples or one gland served as a 'control' for the other. The preparations were not air dried at any time during the procedures. Test slides which were air dried at some stage before the immunological reactions were inferior to their controls either in the morphology of the chromosomes, the background fluorescence levels, the uniformity of the fluorescence staining or a combination of defects.

In situ hybridisation followed, in general, the recipe of Pardue and Gall[7] with modification by Alonzo *et al.*[8] (see legend to Fig. 1). The reaction was effective with 1.0 μg and with 0.2 μg 5S rRNA per slide. It is likely that much smaller amounts could be used if applied in a smaller volume and, perhaps, for longer times[7–9].

The specificity of the immunological reagents in the cytological reaction is demonstrated by the confinement of chromosomal fluorescent label to the 56F region when 5S RNA is included in the *in situ* hybridisation medium (Fig. 1) and by the absence of chromosomal fluorescence when 5S rRNA is omitted (not shown; would be black). Further evidence that the anti-hybrid antibodies are responsible for the chromosomal site of the positive fluorescence reaction is provided by the absence of fluorescence in chromosomes to which 5S RNA had been hybridised but which were treated with antiserum absorbed with poly(rA)·poly(dT) (5 μg poly(rA)·poly(dT) per μl serum for 24 h at 4 °C, centrifuged for 10 min at 6,000*g*). Thus the immunological reagents revealed only DNA RNA hybrids within the nuclei of polytene cells prepared for *in situ* hybridisation.

The specificity was demonstrated further by analyses of the immunological properties of the antiserum. As described previously, several-thousand-fold dilutions of serum reacted in complement fixation assays with poly(rA)·poly(dT), poly(I)·poly(dC) or hybrids of natural RNA and DNA (ref. 1). A 1/50 serum dilution did not react with any single-stranded form of RNA or DNA or with double-stranded RNA or native DNA. When serum was assayed undiluted in counterimmunoelectrophoresis, weak reactions were seen with poly(rA) and with denatured DNA. Both these reactions were eliminated when the serum was passed through a poly(rA) Sepharose affinity column prepared as described by Poonian *et al.*[10] (Fig. 3). With absorbed serum, which gave the same immunofluorescence as unabsorbed serum, the hybrid was the only reactive polynucleotide class even in assays with undiluted serum.

There is [illegible] variable amount of fluorescence in cytoplasm components the origin of which is not yet known. Experiments were projected to attempt to block it while leaving the activity against hybrid nucleic acids intact. Occasional pale fluorescence observed in nucleoli is attributed to contamination of the 5S rRNA probe with fragments of 18S and 28S nucleolar rRNA.

Fig. 1 5S rRNA genes revealed in polytene chromosome 2R of *Drosophila melanogaster* by indirect immunofluorescence detection of RNA–DNA hybrids formed *in situ*. The two homologous 2R chromosomes are not paired except in their most distal portions. The 5S genes (56F on Bridges' standard map) are in the unpaired portions, those derived from one parent to the right, the other parent to the left. In each homologue at least two fluorescent cross-bands are visible. Arrows indicate the 56F regions in the upper photograph of the same chromosomes taken after staining with aceto-orcein. A salivary gland from a fully grown larva of *D. melanogaster* (giant phenotype) was fixed in 50% (v/v) aqueous acetic acid and squashed under a siliconed cover glass, then the slide was frozen on solid CO_2. After snapping off the cover slip, the slide was post-fixed in 3:1 ethanol acetic acid (v/v), rinsed twice in 95% aqueous ethanol (v/v) and stored in 95% ethanol until used. Subsequent treatments were carried out in a moist chamber consisting of a 90-mm square culture dish containing a few leaves of bibulous (or filter) paper saturated with the solvent and two plastic strips to raise the slides above the wet paper. The reagent was placed between the slide and a cover slip. For hybridisation *in situ*, slides were first treated with pancreatic ribonuclease, 100 μg ml^{-1} of 2 × SSC (SSC is 0.15 M NaCl and 0.015 M sodium citrate) for 2 h at 25 °C, then with 90% formamide in 0.1 × SSC for 2 h at 65 °C followed by ice cold 0.1 × SSC rinses. The hybridisation reaction was carried out for 22–24 h at 37 °C in 50% formamide in 4 × SSC using either 1.0 or 0.2 μg 5S RNA per slide highly purified 5S rRNA, extracted from *D. melanogaster* Oregon R embryos[11]. After the annealing reaction, the slides were treated with pancreatic ribonuclease (15 μg ml^{-1}) in 2 × SSC for 2 h at 25 °C, rinsed with phosphate-buffered saline (PBS, 0.14 M NaCl, 0.01 M phosphate, *p*H 7.2) and exposed for 2 h at room temperature (21–23 °C) to rabbit anti-DNA·RNA hybrid serum reconstituted from a lyophilised state by solution in water and diluted for use in PBS (1:20 for Fig. 1). After thorough rinsing in PBS, the slides were finally exposed to a rhodamine-labelled goat IgG fraction of anti-rabbit-IgG (Miles-Yeda) reconstituted to approximately its original concentration, then diluted in PBS for use (1:20 for Fig. 1). The photographs were taken with a microscope equipped with a Zeiss epi-illumination fluorescence module using a 546-nm excitation filter, a 580-nm chromatic splitter and a 580-nm barrier filter for rhodamine fluorescence on 35-mm Eastman Tri X Pan film exposed at ASA 1600 (Diatine developer) at magnifications of approximately × 150 (× 40 objective) or × 370 (× 100 oil immersion objective). Fluorescence exposures were in the range of 1–8 s and phase contrast illumination was adjusted to require approximately the same exposure time so that a single frame could be exposed in both modes simultaneously (not shown here). In some instances, slides were stained with aceto-orcein after fluorescence photography had been completed, then photographed through a No. 58 filter (green) on Plus-X-Pan film at ASA 400. Approx. × 1,000.

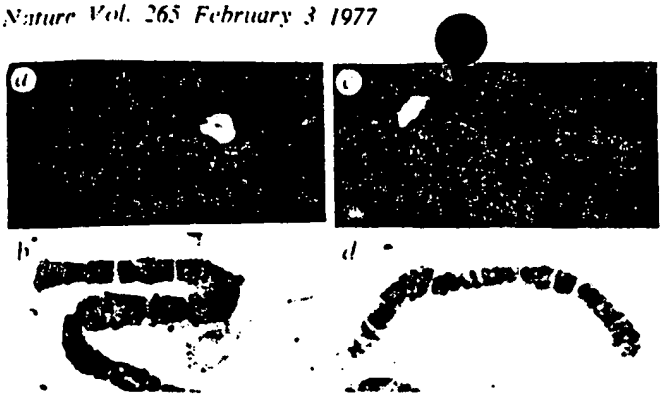

Fig. 2 5S rRNA genes revealed in *D. melanogaster* polytene chromosome 2R as in the legend to Fig. 1. The two chromosomes are unpaired in a single nucleus. The upper chromosome (*a* and *b*) is associated with the nucleolus (nu) at the locus of the 5S genes, fluorescent in (*a*). The homologous chromosome (*c* and *d*) is not visibly associated with the nucleolus; its 5S locus, indicated by the fluorescence in (*c*), is less disperse than that in its homologue (*a*). (*b* and *d*) Photomicrographs of the same fields as in (*a*) and (*c*), respectively, after staining with aceto-orcein. ×630.

but the possibility that 5S DNA templates occur in nucleoli cannot be excluded.

The spatial resolving power of the immunofluorescent probe is equal to that of the optical system used to observe it. Autoradiographs can at best reveal a cluster of silver grains adjacent to, or covering, a labelled region which results in a resolving power of the order of 1–3 μm. So far the precise localisation of the 5S genes within the 56EF region has been equivocal for *D. melanogaster* using autoradiography[1-8]. The images we obtain are of two kinds. In some nuclei, the fluorescence is restricted to a relatively narrow transverse 'band' which is often clearly made up of two subunits and is localised in the 56F region, distal to the puff usually present in 56E (Fig. 1). The possibility that the two subunits reflect the organisation of the 5S locus into the two separable sets of repeated sequences recently reported by Procunier and Tartof[12] is under investigation. On the other hand, the 5S region of chromosome 2R sometimes sticks to the nucleolus as in Fig. 2 (ref. 9) or is ectopically paired to other chromosome regions. In those cases, the fluorescence may be distributed in a network of fibrils extending longitudinally along a much longer segment of chromosome (Fig. 2*a*). When that is true, the morphology in phase contrast and/or after post-staining with aceto-orcein is atypical in that the subsections 56E and F cannot be clearly demarcated and the region does not appear to be organised into distinct bands. Such dispersion of the *in situ* hybrids into fibrils is consistent with the suggestion of Steffensen and Wimber[9] that the 5S genes may have been active in those chromosomes[13]. But the possibility that our 5S probe contains traces of contaminating nucleolar rRNA fragments that could reveal nucleolar rDNA adhering to chromosome 2 has not been entirely excluded.

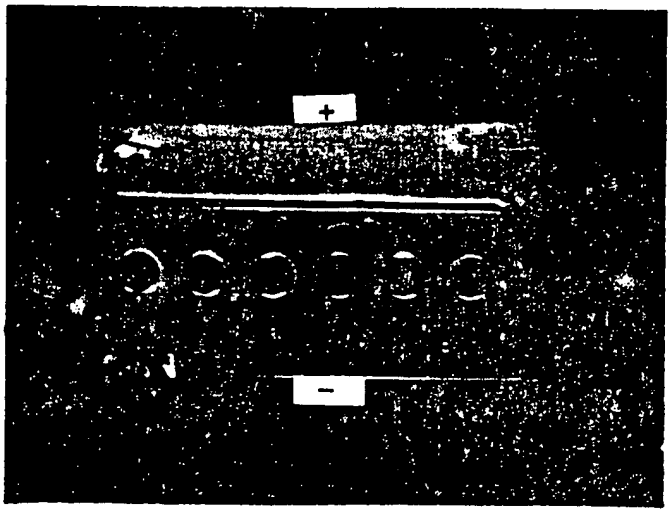

Fig. 3 Specificity of the absorbed antiserum. A sample of 7 ml of anti-poly(rA)·poly(dT) antiserum was absorbed by passage through a 1.2-ml column of poly(rA) Sepharose equilibrated with 0.1 M NaCl, 0.01 M phosphate, *p*H 7.2. The undiluted absorbed serum (200 μl) was placed in the trough; the wells contained 0.5 μg polynucleotide in 50 μl running buffer (0.05 M Tris-HCl, *p*H 8). The polynucleotides were, from left to right: poly(rA); denatured DNA; poly(rA)·poly(dT); poly(dT); poly(I)·poly(C); and poly(dA). The gel medium was 0.8% agar (Difco, purified agar) in running buffer; 7 ml of gel was poured on each 2 × 3 inch glass plate. Electrophoresis was run at 220 V (10 m per plate) for 45 min.

The sensitivity of the technique has not yet been fully explored. At serum dilutions of 1 : 40 for the anti-hybrid rabbit serum and an equivalent concentration for the fluorescent reagent, the fluorescence intensity in the 5S region was very high. Photographic images should still be easily recordable at brightnesses one to two orders of magnitude lower. Thus, the possibility to detect the hybridisation of RNA copies of a unique gene in a polytene chromosome appears to be real. On the other hand, genes present in a size and multiplicity equivalent to the 5S of *D. melanogaster* may be detectable in uninemic chromosomes. An attempt is in progress to detect the 5S locus in human chromosomes at the pachytene stage of meiosis.

The technique is being used to study the distribution of naturally occurring chromosomal RNA detected as hybrid molecules. Polytene chromosomes mounted out of 50% acetic acid display a pattern of fluorescent regions when treated only with the immunological reagents. The regions do not fluoresce if the anti-hybrid serum is blocked with poly(rA)·poly(dT) or if the chromosomes are treated to remove indigenous chromosomal RNA (as in preparation for *in situ* hybridisation), indicating that they are sites of hybrid molecules. Since the locations of the sites change during larval and prepupal development, their RNA moiety could be involved with the control of transcription or of replication or both.

We thank Dr L. Cohen for bringing us together, Dr K. D. Tartof for highly purified *D. melanogaster* 5S RNA, Dr C. R. Alfageme for rhodamine-tagged goat anti-rabbit IgG and for the use of a Zeiss epi-illuminator fluorescence attachment, all three colleagues for useful discussions and technical advice, and Miss D. J. Hazler for technical assistance. This work was supported in part by grants from the NSF and NIH and by an appropriation from the Commonwealth of Pennsylvania.

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Received October 18; accepted December 14, 1976.

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